

# G<sub>M2</sub>-KLH Conjugate Vaccine: Increased Immunogenicity in Melanoma Patients after Administration with Immunological Adjuvant QS-21<sup>1</sup>

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## ABSTRACT

The cell surface gangliosides G<sub>M2</sub>, G<sub>D2</sub>, and G<sub>D3</sub> are often overexpressed in malignant melanoma. We have shown previously that immunization of melanoma patients with G<sub>M2</sub> and *Bacillus Calmette-Guérin* induced an IgM antibody response in most patients and that patients with high titer G<sub>M2</sub> antibodies showed increased survival. As is commonly seen with carbohydrate antigens (which are T independent), the IgM response was short lived, and an IgG response was rarely observed. To increase immunogenicity, we conjugated G<sub>M2</sub> covalently with keyhole limpet hemocyanin (KLH). G<sub>M2</sub>-KLH vaccine was given to melanoma patients alone or with one of the three adjuvants: *Bacillus Calmette-Guérin*, DETOX, or QS-21. The most effective vaccine was G<sub>M2</sub>-KLH with QS-21. It induced a much higher titer, a longer-lasting IgM G<sub>M2</sub> antibody response, and a consistent IgG response (isotype IgG1 and IgG3). It also induced the highest titer anti-KLH response. The results suggest that the conjugate G<sub>M2</sub>-KLH plus QS-21 vaccine elicited significant T-cell help. Because there was no serious toxicity, this vaccine approach is attractive for augmenting the immunogenicity of other gangliosides, such as G<sub>D2</sub> and G<sub>D3</sub>, and to determine the effects of ganglioside antibodies on the course of melanoma. In addition, the finding that QS-21 significantly increased the immunogenicity of G<sub>M2</sub>-KLH suggests that it may do the same for other conjugate vaccines, many of which are currently used without adjuvant.

## INTRODUCTION

One of the changes that occur in the process of malignant transformation is an altered pattern of cell surface ganglioside expression in certain types of cancer, including malignant melanoma (1). In normal melanocytes, G<sub>M3</sub><sup>3</sup> is the predominant ganglioside. Other gangliosides, which include G<sub>D3</sub>, G<sub>M2</sub>, G<sub>D1a</sub>, and G<sub>T1b</sub>, constitute less than 10% of the total (2). In malignant melanoma, activation of glycosylating enzymes leads to increased expression of G<sub>D3</sub>, G<sub>D2</sub>, G<sub>M2</sub>, and 9-O-acetyl G<sub>D3</sub> (3, 4). These overexpressed gangliosides are attractive targets for immunotherapy, including active immunization with ganglioside vaccines. In a series of studies involving G<sub>M2</sub> vaccines in patients with malignant melanoma, we have shown that vaccination (after low-dose cyclophosphamide and with BCG as adjuvant) induces IgM antibodies to G<sub>M2</sub> in most patients (5), and that disease-free interval and survival are extended in patients producing

high-titer G<sub>M2</sub> antibodies (6, 7). However, the induced antibody response to G<sub>M2</sub> has the characteristics of a T-independent response (predominantly IgM, short duration, inconsistent IgG response, and lack of booster effect), and the other melanoma gangliosides, G<sub>D3</sub> and G<sub>D2</sub>, are not immunogenic when administered in the same way (8). As the relevant epitopes are carbohydrates, we have explored approaches to increasing immunogenicity that are suggested by the successful development of carbohydrate vaccines for bacterial infections. In the mouse, we have shown that the immunogenicity of G<sub>D3</sub> is markedly increased by covalent binding to KLH and that mice immunized with the G<sub>D3</sub>-KLH conjugate and the adjuvant QS-21 show a high-titer IgM response, followed by a strong, long-lasting IgG response (9). We have now begun to test ganglioside conjugate vaccines in melanoma patients and report here the results of initial studies with vaccines containing G<sub>M2</sub>-KLH conjugate plus various adjuvants.

## MATERIALS AND METHODS

### Patients

Forty-eight patients with malignant melanoma stage III or stage IV who were free of detectable disease as a consequence of surgery within the previous 4 months were treated. None of the patients had received prior chemotherapy or radiation therapy. Six of the 30 patients receiving G<sub>M2</sub>-KLH plus QS-21 were part of an initial Phase I study and have been described previously (10).

### Vaccine Preparation and Administration

**G<sub>M2</sub>-KLH Vaccine.** G<sub>M2</sub>-KLH conjugate was prepared by Biomira, Inc. and in our laboratory as described previously for G<sub>D3</sub>-KLH conjugate vaccine (9). Briefly, the conjugation procedure involved ozone cleavage of the ceramide double bond of G<sub>M2</sub>, introduction of an aldehyde group, and conjugation to aminolysyl groups of KLH by reductive amination. The G<sub>M2</sub>-KLH molar ratio was approximately 800:1, and one patient dose contained 70 µg G<sub>M2</sub> and approximately 500 µg KLH in 0.5 ml of normal saline. Groups of six patients each received G<sub>M2</sub>-KLH conjugate without adjuvant, G<sub>M2</sub>-KLH with DETOX, and G<sub>M2</sub>-KLH with BCG, and thirty patients received G<sub>M2</sub>-KLH with QS-21.

Four vaccinations were administered intradermally into extremities with intact lymphatic drainage at 2-week intervals, followed by two additional vaccinations at 8-week intervals. Cyclophosphamide (Cytoxan; Mead Johnson and Co., Evansville, IN; 200 mg/m<sup>2</sup>) was administered i.v. to all patients 4 to 6 days before the first vaccination.

**Immunological Adjuvants.** DETOX was produced and supplied by Ribi Immunochem Research, Inc. (Hamilton, MT) formulated as a lyophilized oil droplet emulsion. It consists of CWS from BCG and MPLA from *Salmonella minnesota* R595. On the day of vaccination, 0.25 ml DETOX (250 µg CWS + 25 µg MPLA) was mixed with the G<sub>M2</sub>-KLH preparation. The vaccine (final volume, 0.75 ml) was vortexed for 2–3 min and administered to the patients within 15 min. BCG was purchased from Bionetics Research, Inc. (Rockville, MD). On the day of vaccination, 10<sup>7</sup> viable units of BCG in 0.1 ml normal saline were added to the G<sub>M2</sub>-KLH vaccine in each individual syringe (final volume, 0.6 ml). The contents were mixed and administered to the patients within 15 min. QS-21 adjuvant (a homogeneous saponin purified from the bark of *Quillaja saponaria* Molina; Refs. 11 and 12) was provided by Cambridge Biotech, Inc. (Worcester, MA). QS-21 (100 or 200 µg) were diluted in 0.25 ml normal saline and mixed with G<sub>M2</sub>-KLH. The vaccine (final volume, 0.75 ml) was vortexed for 2–3 min and administered within 15 min.

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<sup>3</sup> The abbreviations used, in accordance with the ganglioside nomenclature proposed by Svennerholm (36), are: G<sub>M3</sub>, 1<sup>3</sup>NeuAc-GalCer; G<sub>M2</sub>, 1<sup>3</sup>NeuAc-GgOse<sub>3</sub>Cer; G<sub>M1</sub>, 1<sup>3</sup>NeuAc-GgOse<sub>2</sub>Cer; G<sub>D3</sub>, 1<sup>3</sup>(NeuAc)<sub>2</sub>GgOse<sub>3</sub>Cer; G<sub>D2</sub>, 1<sup>3</sup>(NeuAc)<sub>2</sub>-LacCer; G<sub>D1a</sub>, 1<sup>3</sup>NeuAc, 1<sup>3</sup>NeuAc-GgOse<sub>3</sub>Cer; G<sub>D1b</sub>, 1<sup>3</sup>(NeuAc)<sub>2</sub>-GgOse<sub>3</sub>Cer; and G<sub>T1b</sub>, 1<sup>3</sup>(NeuAc)<sub>2</sub>(N<sup>3</sup>)(NeuAc)GgOse<sub>3</sub>Cer. Other abbreviations used are: BCG, *Bacillus Calmette-Guérin*; KLH, keyhole limpet hemocyanin; CWS, cell wall skeletons; MPLA, monophosphoryl lipid A; Hib, *Haemophilus influenzae* type b; PRP, phosphoribosyl/nititolphosphate polysaccharide; HPTLC, high performance thin layer chromatography.

## Gangliosides

G<sub>M2</sub> from bovine brain was received from Fidia Research Laboratory (Abano Terme, Italy) or was isolated from Tay-Sachs cat brains in our laboratory by published procedures. G<sub>M3</sub>, G<sub>M1</sub>, G<sub>D1a</sub>, and G<sub>D1b</sub> from bovine brain were purchased from Sigma Chemical Co. (St. Louis, MO). Asialo-G<sub>M2</sub> was prepared by treatment of G<sub>M2</sub> with 0.1 M trifluoroacetic acid at 100°C for 1 h, followed by separation on a reversed phase column (Sep-Pak C<sub>18</sub>; Waters, Milford, MA). G<sub>D2</sub> was made from G<sub>D1b</sub> by treatment with  $\beta$ -galactosidase. G<sub>D3</sub> was isolated from bovine buttermilk and kindly provided by Dr. R. K. Yu (Medical College of Virginia, Richmond, VA).

## Reagents and Monoclonal Antibodies

HPTLC silica gel plates were obtained from E. Merck (Darmstadt, Germany); 4-chloro-1-naphthol and *p*-nitrophenyl phosphate disodium were obtained from Sigma. Alkaline phosphatase-conjugated goat anti-human IgM (Kierkegaard and Perry Labs, Gaithersburg, MD) and mouse anti-human IgG (Southern Biotech, Birmingham, AL), followed by alkaline phosphatase-conjugated goat anti-mouse IgG (Southern Biotech), were used for ELISA. Horseradish peroxidase-conjugated goat anti-human IgM or IgG purchased from TAGO (Burlingame, CA) was used for dot blot immune stain and immune thin layer chromatography. Rabbit anti-mouse immunoglobulins conjugated to horseradish peroxidase for immune thin layer chromatography and rabbit anti-mouse IgM and IgG conjugated to alkaline phosphatase for ELISA were used with control monoclonal mouse antibodies and were obtained from Zymed (San Francisco, CA). Murine anti-G<sub>M2</sub> mAb 696 (IgM) was kindly provided by Kyowa Hakko Kogyo Co., Ltd. (Tokyo, Japan; Refs. 13 and 14), and anti-G<sub>D3</sub> mAb R24 (IgG3) was generated in our laboratory (15).

## Serological Assays

ELISA was performed as described previously (6). To control for nonspecific "stickiness," immune sera were also tested on plates that were processed identically but to which no ganglioside had been added, and the reading was subtracted from the value obtained in the presence of ganglioside. The titer was defined as the highest dilution yielding a corrected absorbance of 0.1 or greater. Immunostaining of gangliosides with mAbs or human sera was performed after spotting on nitrocellulose strips (16) or separation on HPTLC silica gel glass plates as described previously (3). Plates were developed in chloroform:methanol:water (0.25% CaCl<sub>2</sub>) 50:40:10 (v/v), and gangliosides were visualized by staining with resorcinol/HCl reagent or mAbs.

## Determination of IgG Subclass

Determination of IgG subclass was performed by ELISA using subclass-specific secondary mouse anti-human IgG1, IgG2, IgG3, and IgG4 mAbs. Secondary mAbs from different suppliers (Table 2) were used. Alkaline phosphatase conjugated to goat anti-mouse IgG (Southern Biotech) was used as third antibody at a dilution of 1:200.

## Complement-mediated Cytotoxicity Assays

Complement-mediated cytotoxicity assays were performed by a 4-h <sup>51</sup>Cr release assay. Cells from the G<sub>M2</sub>-positive melanoma cell line SK-MEL-173 served as target cells. Cells (2 × 10<sup>6</sup>) were labeled with 100  $\mu$ Ci Na<sub>2</sub><sup>51</sup>CrO<sub>4</sub> (New England Nuclear, Boston, MA) in 10% FCS RPMI for 1 h at 37°C in a CO<sub>2</sub> incubator. The cells were washed twice, and 10<sup>4</sup> cells/well in 96-well round-bottomed plates (Corning, New York, NY) were labeled and incubated with 1:5 diluted pre- or postvaccination serum or with medium alone for 1 h at 37°C in a CO<sub>2</sub> incubator. The cells were washed and incubated with human complement (Sigma) at a dilution of 1:4 for 4 h at 37°C. The plates were spun at 500 × g for 5 min, and an aliquot of 125  $\mu$ l of supernatant of each well was harvested for determination of released <sup>51</sup>Cr. All assays were performed in triplicate and included control wells for maximum release in 1% NP40 (Sigma) and for spontaneous release in the absence of complement. The percentage of specific lysis was calculated as follows:

$$\% \text{ cytotoxicity} = \frac{\text{Experimental release} - \text{spontaneous release}}{\text{Maximum release} - \text{spontaneous release}} \times 100$$

## Statistical Analysis

Titers between groups were compared using the Wilcoxon rank-sum test. Because the purpose of the study was to generate rather than to test hypotheses, the *P*s were not adjusted for multiple comparison.

## RESULTS

**Vaccine Administration and Side Effects.** Forty-eight patients were immunized with the G<sub>M2</sub>-KLH vaccine. Groups of 6 patients each received G<sub>M2</sub>-KLH with no immunological adjuvant or with DETOX and BCG, and 30 patients received G<sub>M2</sub>-KLH with QS-21. No local or systemic toxicity was detected after administration of G<sub>M2</sub>-KLH alone. Vaccines containing DETOX resulted in nodule formation at vaccination sites in four of six patients that lasted 2–10 weeks. In four patients, these were associated with 3–10 cm of erythema and induration but only minimal tenderness. In one patient, it was associated with 25 cm erythema and induration after one immunization, and in a second patient, low grade fever and malaise for 72 h after the first immunization. In this patient, the DETOX dose was reduced to 50  $\mu$ g CWS + 5  $\mu$ g MPLA for the subsequent immunizations. BCG produced local inflammation and crusting at some point in all patients, which healed after 2–12 weeks. When this occurred, the dose of BCG was reduced from 1 × 10<sup>7</sup> viable units to a final dose of 3 × 10<sup>6</sup> units in four patients and 1 × 10<sup>6</sup> units in one patient. The sixth patient had a history of tuberculosis exposure and a positive purified protein derivative (PPD) test and was, therefore, started at a dose of 1 × 10<sup>6</sup> units, which was eventually reduced to 1 × 10<sup>5</sup> units. QS-21 induced mild local erythema, induration, and tenderness lasting 24–72 h in all patients at the 100- $\mu$ g dose. The 200- $\mu$ g dose of QS-21 was associated with local tenderness and inflammation lasting 2–10 days in all patients as well as mild flu-like symptoms, including low grade fever (<38.5°C), headache, and myalgia lasting 8–24 h after most immunizations. No neurological abnormalities or other side effects were observed.

**Antibody Response to G<sub>M2</sub>-KLH Conjugate Vaccines.** Before vaccination, IgG antibodies against G<sub>M2</sub> were not detected, and IgM antibodies were detected only rarely. IgM titers of 1:40 were seen in three patients, and two patients had a pretreatment titer of 1:320. The remaining 43 patients showed G<sub>M2</sub> reactivity with 1:20 titers or lower before vaccination. ELISA and immune stain results with sera obtained before and after immunization are summarized in Table 1. The IgM antibody titers after immunization with G<sub>M2</sub>-KLH or with G<sub>M2</sub>-KLH and DETOX or BCG were quite similar (median titer, 1:80–1:240; *P* > 0.15 between any pair of groups). In contrast, 25 of 30 patients immunized with G<sub>M2</sub>-KLH and QS-21 showed IgM antibody titers of 1:320 or more, significantly higher than the titers in the other groups (*P* < 0.01, *P* = 0.02, and *P* = 0.06) or in patients immunized with previous G<sub>M2</sub>/BCG vaccines (*P* < 0.001; Ref. 6). In addition, immunization with G<sub>M2</sub>-KLH and QS-21 induced a consistent IgG response for the first time; only 5 of the other 18 patients receiving G<sub>M2</sub>-KLH vaccines produced comparable IgG titers.

Median sequential IgM and IgG antibody titers against G<sub>M2</sub> in patients receiving G<sub>M2</sub>-KLH alone or with adjuvants DETOX and BCG and the first six patients of the G<sub>M2</sub>-KLH plus QS-21 group are shown in Fig. 1. IgM peak titers were seen after the third or fourth vaccination and remained elevated in most patients receiving the QS-21 vaccine for at least 20 weeks. Booster immunizations at weeks 14 and 22 did not further increase IgM titers. IgG titers of 1:160 or higher were seen 2 weeks after the fourth vaccination in five of six patients receiving the QS-21 vaccine. The titers decreased to 1:40 or less but rapidly increased again after booster vaccination to the previous levels (median 1:160) and remained at this level for more

Table 1 Serological response of patients receiving  $G_{M2}$ -KLH conjugate vaccines with or without adjuvants in comparison to vaccine containing  $G_{M2}$  adherent to BCG ( $G_{M2}$ /BCG)

Vaccine	No. of patients	Reciprocal G <sub>M2</sub> antibody titers				Dot blot immune stain for G <sub>M2</sub> antibodies		Reciprocal KLH antibody titers after immunization (peak) IgG
		Before immunization		After immunization (peak)		IgM	IgG	
		IgM	IgG	IgM	IgG			
G <sub>M2</sub> -KLH Median titers	6	10(2),0(4) 0	0(6) 0	320,160,80(2),40,20 80	10,0(5) 0	3*(2),2*(2),0(2)	1*(2),0(4)	360,180(3),20(2) 180
G <sub>M2</sub> -KLH + DETOX Median titers	6	20(2),10(2),0(2) 10	0(6) 0	640(2),160(3),40 160	160,0(5) 0	3*(2),2*(3),1*	2*,0(5)	1080(2),360,180(3) 270
G <sub>M2</sub> -KLH + BCG Median titers	6	40,20,0(4) 0	0(6) 0	1280,320(2),160(2),40 240	320,20(2),0(3) 10	3*2*(3),0(2)	1*(2),0(4)	3240,1080(2),360(2),180 720
G <sub>M2</sub> -KLH + QS-21 Median titers	30	320(2),40(2) 20(11),0(15) 10	0(30) 0 0	5120(2),1280(13),640(4) 320(6),160(3),80(2) 960	1280(2),640(5),320(5) 160(8),80(2),40(5),20,10(2) 160	3*(28),2*(2)	3*(16),2*(5) 1*(3),0(6)	7290(4),*2430,810 7290 ND
G <sub>M2</sub> /BCG <sup>a</sup> Median titers	58	160,40,20(10), 10(11),0(35) 0	0(58) 0 0	640(11),320(9), 160(15),80(8),40(8) 20(2),10(3),0(2) 160	640,160,80(4), 20,10(3),0(48) 0	3*(32),2*(15), 1*(4),0(7)	3*(2),2*(5), 1*(4),0(47)	7290 ND ND

<sup>a</sup> Only the first six patients were analyzed for anti-KLH antibodies. ND, Not done.

<sup>a</sup> Only the first six patients were analyzed for anti-KLH antibodies. ND, not done.

<sup>b</sup> Historical data (6).

than 11 weeks. The second booster vaccination had no clear effect on antibody titers in most cases. Thus, the response to booster vaccination showed only one of two characteristics of the classical secondary immune response. The response occurred more rapidly, but antibody titers did not rise higher than after the initial immunization.

KLH antibodies were not detected in pretreatment sera. After vaccination, all patient sera showed reactivity with KLH as indicated in Table 1. The highest titers of IgG antibodies were seen after administration with QS-21 (the first six patients were tested), significantly higher than in all other groups, including the next-best group of patients vaccinated with  $G_{M2}$ -KLH and BCG ( $P = 0.006$ ). In the QS-21 group, there was no correlation between the strength of the  $G_{M2}$  response and the KLH response.

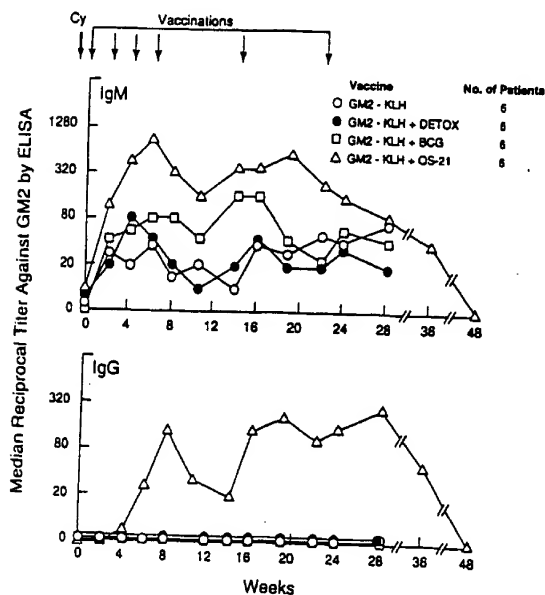


Fig. 1. Median IgM and IgG antibody titers over time in groups of six melanoma patients after immunization with  $G_{M2}$ -KLH alone or  $G_{M2}$ -KLH plus immunological adjuvants DETOX, BCG, and QS-21. Arrows, time of vaccine injections.

**Specificity Analysis of  $G_{M2}$  Antibodies.** The specificity of ganglioside antibodies detected in the patients' sera before and after immunization was determined by dot blot immune stains using the ganglioside standards  $G_{M3}$ , asialo- $G_{M2}$ ,  $G_{M2}$ ,  $G_{M1}$ ,  $G_{D2}$ ,  $G_{D3}$ ,  $G_{D1a}$ , and  $G_{D1b}$  (Fig. 2; first six patients of  $G_{M2}$ -KLH plus QS-21 group are shown). Preimmunization IgM and IgG antibodies from most patients showed weak reactivity with asialo- $G_{M2}$ , and some patients also had IgM antibodies against  $G_{M1}$  and  $G_{D1b}$ . Reactivity with these gangliosides was not altered by immunization. The only vaccine-induced changes were strong reactivity with  $G_{M2}$  and weak reactivity with  $G_{D2}$ . Dot blot immune stains were graded as 0, 1\*, 2\*, or 3\*. Reactivity of 3\* for IgM antibodies against  $G_{M2}$  was seen in the serum of 28 of 30 patients immunized with  $G_{M2}$ -KLH and QS-21, in 1 of 6 patients treated with  $G_{M2}$ -KLH and BCG, and in 2 of 6 patients treated with  $G_{M2}$ -KLH without adjuvant or  $G_{M2}$ -KLH and DETOX. Reactivity of 3\* for IgG antibodies was seen in 16 of 30 patients immunized with  $G_{M2}$ -KLH and QS-21 and in none of the patients in the other treatment groups.

Postvaccination sera from the first six patients immunized with  $G_{M2}$ -KLH and QS-21 were also tested by immune thin layer chromatography (Fig. 3) for: reactivity with  $G_{M2}$  and other gangliosides of a melanoma tissue extract. Most patients' sera showed strong IgG and IgM reactivity with  $G_{M2}$  isolated from bovine brain or melanoma. Antisera reactivity was seen also with a lower migrating band in melanoma extract, presumably  $G_{D2}$ .

To confirm the  $G_{D2}$  cross-reactivity of IgG antibodies, postvaccination serum from patient no. 2 was preincubated with either  $G_{M2}$  or  $G_{D2}$  before performing the immune stain (Fig. 4). Reactivity with  $G_{M2}$ , and with  $G_{D2}$  in the melanoma ganglioside extract, was completely inhibited by preincubation with  $G_{M2}$ . On the other hand, preincubation of the same serum with  $G_{D2}$  resulted in inhibition of  $G_{D2}$  reactivity only and did not change reactivity with  $G_{M2}$ . These results suggest the presence of two populations of antibodies, one reacting with  $G_{M2}$  alone and another with reactivity for  $G_{M2}$  and  $G_{D2}$ .

**Subclass Determination of IgG Antibodies.** IgG sera from the first six patients immunized with  $G_{M2}$ -KLH and QS-21 were tested by ELISA using a panel of IgG subclass-specific secondary antibodies. The results are summarized in Table 2. The IgG antibodies in all six sera tested were of IgG1 and IgG3 subclass.

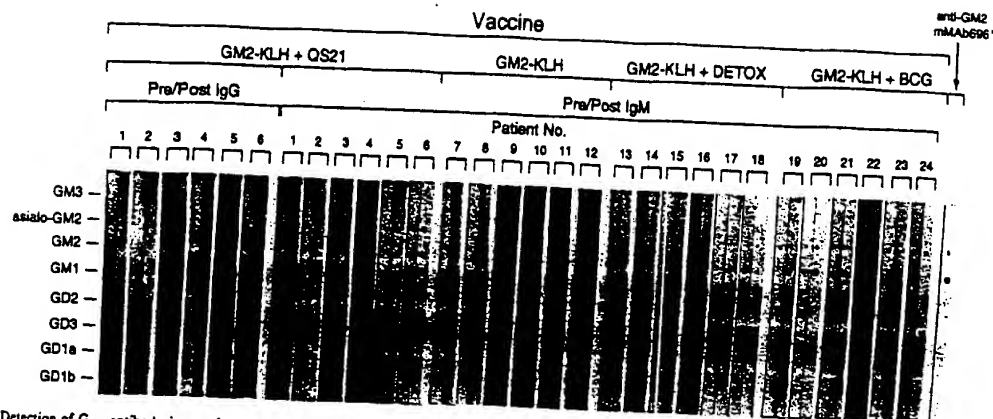


Fig. 2. Detection of  $G_{M2}$  antibody in sera from patients vaccinated with  $G_{M2}$  conjugate vaccine plus adjuvant by dot blot immune staining. Ganglioside standards were spotted on nitrocellulose strips (ordinate) and allowed to react with prevaccination and peak titer postvaccination sera from individual patients and peroxidase-labeled goat anti-human IgM or IgG antibody. Strips are graded on a scale from 0 to 3+. mAb 696 was used as positive control for  $G_{M2}$ .

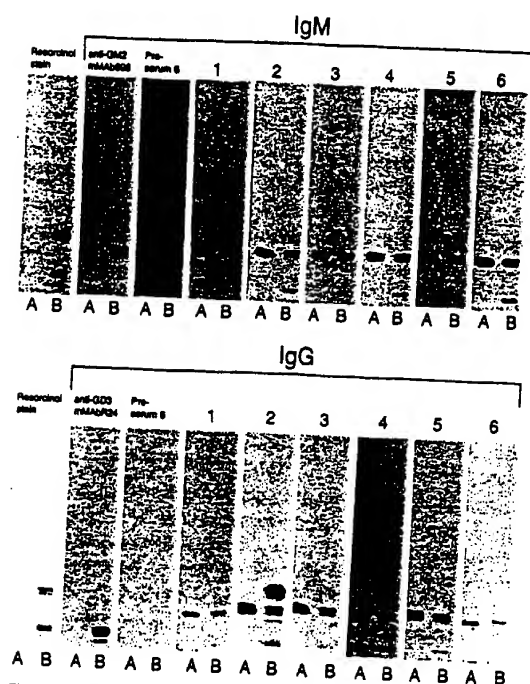


Fig. 3. Specificity of peak titer sera from patients immunized with  $G_{M2}$ -KLH + QS-21 vaccine determined by immune thin layer chromatography as described previously (3).  $G_{M2}$  (A) and melanoma tissue ganglioside extract (B) were applied to HPTLC plates, incubated with sera from individual patients, and stained with peroxidase-labeled goat anti-human IgM or IgG antibody. mAb 696 was used as positive control for  $G_{M2}$  and resorcinol stain for gangliosides.

**Complement-mediated Cytotoxicity.** Effector function of anti- $G_{M2}$  antibodies in the serum of the first six patients vaccinated with  $G_{M2}$ -KLH and QS-21 (diluted 1:5) was tested by complement-mediated cytotoxicity assays. As shown in Table 3, postvaccination sera of all six patients lysed  $G_{M2}$ -positive SK-MEL-173 melanoma cells in the presence of human complement, and prevaccination sera were not cytotoxic with complement, and postvaccination sera were not cytotoxic when complement was not added. More detailed study of

cell surface binding and cytotoxic effector functions of vaccine-induced antibodies and their subclasses is under way.

## DISCUSSION

In a series of studies in patients with malignant melanoma, one objective has been to construct vaccines that are effective in inducing production of antibodies against three gangliosides often overexpressed in melanoma:  $G_{M2}$ ,  $G_{D2}$ , and  $G_{D3}$ . Our initial approach was to vaccinate patients with unconjugated gangliosides adsorbed to BCG. In this way, we were able to induce antibody production against  $G_{M2}$  (5, 6) but not  $G_{D2}$  or  $G_{D3}$ .  $G_{M2}$  antibodies induced by  $G_{M2}$ /BCG vaccines were mostly of the IgM class, the antibody response was of short duration, and booster immunization resulted again in a brief period of IgM antibody production similar to the primary response; all characteristics of a T-cell-independent immune response, well known from studies of other carbohydrate antigens. Even so, vaccine-induced production of  $G_{M2}$  antibodies by patients with stage III melanoma after surgery was associated with increased survival (6, 7). This

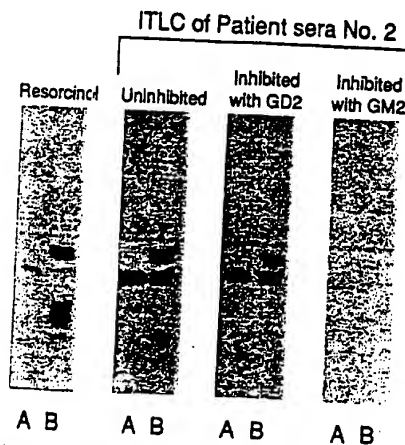


Fig. 4. Inhibition of IgG reactivity of patient serum against  $G_{M2}$  and  $G_{D2}$ .  $G_{M2}$  (A) and melanoma tissue ganglioside extract (B) were applied to HPTLC plates, incubated with serum from patient no. 2, and stained with peroxidase-labeled goat anti-human IgG antibody. Patient serum (3 ml) at a dilution of 1:50 was preincubated (inhibited) with either 150  $\mu$ g  $G_{M2}$  or 150  $\mu$ g  $G_{D2}$  prior to immune staining.

observation suggested that melanoma gangliosides are appropriate candidates for vaccine construction and that melanoma ganglioside vaccines of increased immunogenicity might result in superior clinical outcomes. Because the relevant epitopes of melanoma gangliosides are carbohydrates, it is helpful to consider what efforts have been made in attempts to increase the immunogenicity of carbohydrate vaccines, notably against certain bacterial infections.

The major distinction of the immune response to carbohydrate antigens, as opposed to protein antigens, is that it does not depend on the thymus. The concept that carbohydrate antigens are thymus independent is based on the observation that neonatally thymectomized mice as well as athymic mice show unimpaired humoral immune responses to bacterial polysaccharides (17). B cells that respond to thymus-independent antigens show several characteristic features. They appear later in ontogeny, are long-lived, and do not require T cells for activation, at least not *in vivo*. Although T cells are required for B cells to respond to thymus-independent antigens *in vitro*, the nature of the T-cell effect is poorly understood and clearly different from the MHC-restricted T-cell help in the T-dependent antibody response to protein antigens. Although T-cells are not indispensable for the *in vivo* antibody response to thymus-independent antigens, antibody levels are higher when T cells are present, suggesting a general augmenting activity of T cells, again by unknown mechanisms (18).

A large variety of approaches has been explored in attempts to increase the immunogenicity of carbohydrate antigens. They include chemical modification (19), administration with adjuvants, noncovalent complexing with proteins, covalent attachment to immunogenic protein carriers (20), and replacement of the carbohydrate epitope by a protein replica, either peptides synthesized *de novo* (so-called mimotopes; Ref. 21) or antiidiotype antibodies (22). Most of these approaches result in increased T-cell help for the carbohydrate-specific antibody response. While each has shown promise in initial experimentation, covalent attachment of carbohydrate antigens to immunogenic T-dependent protein carriers, as first suggested for haptens (23) and then disaccharides (24), is the concept that has been pursued most vigorously, resulting in vaccines that have in some instances been shown to be highly effective in recent clinical trials.

Excellent examples are Hib polysaccharide protein conjugate vaccines. Four vaccines that have been developed over the last decade differ in the carbohydrate compounds, the protein carriers, and the linkers between carbohydrate and protein (25-29). In comparative

Table 3 Complement lysis of melanoma cell line SK-MEL-178 mediated by  $G_{M2}$  antibodies in sera from patients immunized with  $G_{M2}$ -KLH plus QS-21

Patient no.	Percentage of lysis <sup>a</sup>		
	Prevaccination serum with complement	Postvaccination serum with complement	Postvac. serum without complement
1	1.3	38.75	3.1
2	2.2	16.9	2.7
3	1.1	14.0	0.9
4	1.2	26.0	2.2
5	2.1	34.9	1.7
6	10.5	44.7	2.3

<sup>a</sup> Target cells were labeled with  $^{51}\text{Cr}$  and treated with 1:5 diluted antisera. Vac., vaccination.

studies in children, all conjugate vaccines induced a much stronger antibody response than unconjugated Hib PRP vaccine (30). Of particular interest are observations that young children first immunized with HbOC (oligosaccharide-nontoxic diphtheria toxin) or PRP-OMPC (outer membrane protein complex of *Neisseria meningitidis* type B) vaccines and later challenged with unconjugated PRP vaccine showed an anamnestic IgG response, even if challenged at an age at which they do not respond to primary immunization with the unconjugated vaccine (31, 32). How T cells are engaged and how they interact with Hib PRP-responsive B-cells is still far from clear. The fact that increased immunogenicity and T dependence require a covalent bond between PRP and protein suggests that the proximity between protein and PRP must not be disturbed, at least not in the early phase of antigen processing. As the isotype and biological activities of antibodies induced by Hib PRP and Hib PRP conjugates are the same, it appears that the B cells that respond to the conjugate-induced T-cell signal are qualitatively identical with those engaged by Hib PRP alone. Drawing on the substantial experience that has accumulated in the development of carbohydrate vaccines for bacterial infections, we have explored, over the past 5 years, similar approaches in our attempts to increase the immunogenicity of melanoma gangliosides. Chemical modification of  $G_{D3}$ , resulting in lactone, amide, or gangliosidol formation, produced derivatives that were highly effective in inducing antibody production. However, the antibodies induced by  $G_{D3}$  lactone,  $G_{D3}$  amide, or  $G_{D3}$  gangliosidol did not cross-react with  $G_{D3}$  (33, 34). An antiidiotype antibody BEC-2, mimicking  $G_{D3}$ , was developed by immunizing mice with the monoclonal antibody R24, which recognizes  $G_{D3}$ . Rabbits immunized with BEC-2 produced anti- $G_{D3}$  antibodies (35), and initial studies of the immunogenicity of BEC-2 in human patients are under way.

Regarding conjugate vaccines, our initial studies with  $G_{D3}$  in the mouse were concerned with three issues: development of the conjugation method; selection of the carrier protein; and choice of the adjuvant (9). The optimal conjugation procedure involved ozone cleavage of the double bond of  $G_{D3}$  in the ceramide backbone, introduction of an aldehyde group, and coupling to protein aminolysyl groups by reductive amination. Of five carriers tested, poly-L-lysine, KLH, cationized BSA, *Neisseria meningitidis* outer membrane protein complex, and multiple antigenic peptide containing four repeats of a malarial T-cell epitope. KLH was found to be most effective. Noncovalent  $G_{D3}$ /KLH complexes were not immunogenic. The best adjuvant was QS-21, a homogeneous saponin fraction purified from the bark of *Quillaja saponaria* Molina. The characteristics of the antibody response to immunization with  $G_{D3}$ -KLH conjugate and QS-21 included: (a) a high initial antibody titer; (b) a rapid secondary rise of IgM antibody titers after booster immunizations; (c) maintenance of IgM antibody titers after booster immunizations for up to 10 weeks; and (d) consistent production of IgG antibody at high titers, parallel to IgM antibody production, except for the initial delay of 2 weeks. These findings have now been reproduced in human melanoma pa-

Table 2 Characterization of IgG antibodies induced against  $G_{M2}$  with  $G_{M2}$ -KLH plus QS-21 vaccine by IgG subclass-specific mAbs

IgG subclass mAbs			Reciprocal ELISA titer against $G_{M2}$					
Specificity	Conc. <sup>a</sup> (μg/ml)	mAbs source <sup>b</sup>	Patient no.					
IgG	10	SBA <sup>a</sup>	640	640	640	640	640	640
	20	BS	40	20	20	n.d.	20	20
	5	ZLI	640	640	640	640	640	640
IgG1	10	SBA	10	10	10	10	10	10
	20	BS	10	10	0	10	0	0
	2	ZLI	10	0	10	0	10	0
IgG2	10	SBA	10	0	0	0	0	0
	20	BS	0	0	0	0	0	0
	2	ZLI	0	0	0	0	0	0
IgG3	10	SBA	20	20	20	20	20	20
	20	BS	40	80	40	40	40	40
	2	ZLI	20	40	20	10	10	20
IgG4	10	SBA	0	0	0	0	0	0
	20	BS	0	0	0	0	0	0
	2	ZLI	0	0	0	0	0	0

<sup>a</sup> Conc., concentration.

<sup>b</sup> SBA, Southern Biotechnology Associates (Birmingham, AL); BS, The Binding Site, Ltd.; ZLI, Zymed Laboratories, Inc. (San Francisco, CA).

tients by immunization with another ganglioside conjugate vaccine,  $G_{M2}$ -KLH, using the same conjugation procedure. As in the mouse studies, QS-21 proved to be a significantly more effective adjuvant than DETOX or BCG, with acceptable toxicity.

The  $G_{M2}$  antibody response had many characteristics of a T-cell-dependent response. It was long-lasting, and antibodies of IgG1 and IgG3 subclass (usually associated with a T-cell-dependent immune response) were induced. As seen with the Hib-PRP vaccines, these isotypes were the same as those induced occasionally at low titers with unconjugated  $G_{M2}$ -BCG vaccines. The lack of a clear booster effect in the sustained high-titer IgM and IgG response after vaccinations 3 and 5 months following the initial series may be explained by the fact that the patients were immunized at 2-week intervals initially. In the classical experiment showing the secondary response to protein antigens, the second injection of antigen is given 4 weeks after the first. Antibody levels after the first immunization are higher between 1 and 2 weeks after the injection and then decline to very low levels before the booster injection is given after 4 weeks. In the immunization schedule we chose, the initial antibody response did not subside but increased in a stepwise fashion in response to the first four vaccinations at the 2-week intervals, anticipating the secondary response that is seen in a more dramatic fashion in the classical experiment. Unlike the antibody response to most protein antigens, the IgM response was long-lasting, and IgM antibodies remained at higher titer than IgG antibodies, even after repeated booster immunizations, as is characteristic for carbohydrate antigens. Hence, the immune response against gangliosides that contain a comparably short oligosaccharide chain linked to a lipid backbone and that are autoantigens show much in common with the immune response against Hib-PRP and other bacterial carbohydrates.

The development of the  $G_{M2}$ -conjugate vaccine will make it possible to determine whether higher levels of IgM and IgG antibodies against  $G_{M2}$ , sustained over longer periods, will be more effective in delaying recurrence of melanoma than the lower levels of mostly IgM antibodies, present for shorter periods, in patients immunized with unconjugated  $G_{M2}$ . In addition, we can now test whether conjugation with immunogenic protein carriers also confers immunogenicity to  $G_{D3}$  and  $G_{D2}$ , major gangliosides which have not induced a consistent antibody response in melanoma patients when given as unconjugated vaccines. If this can be accomplished, construction and testing of a polyvalent melanoma ganglioside vaccine would be an attractive next step.

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